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Journal of Chromatography A, 932 (2001) 33–41

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Application of matrix solid-phase dispersion in the determination of dibenzo[*a*,*l*]pyrene content of experimental animal diets used in a large-scale tumor study

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Received 18 June 2001; accepted 8 August 2001

Abstract

A method utilizing matrix solid-phase dispersion (MSPD) was developed for isolation and determination of dibenzo[*a*,*l*]pyrene (DBP) in experimental rainbow-trout diets used in a large-scale carcinogenesis study. A 0.5 g sample of moist ration containing 0–225 ppm DBP (dry basis) was mixed with 2 g C_{18} sorbent and benzo[*a*]pyrene internal standard was added to the mixture. Extraction and clean-up were accomplished in a single step by extracting the sample mixture with hexane–benzene 4:1 from a cartridge containing 2 g Florisil®. DBP was quantified by HPLC on a C_8 bonded phase column with fluorescence detection. Mean analytical recovery of DBP from control diet spiked at three concentration levels was 101 to 107% with relative standard deviations of 1 to 7%. The limit of detection of DBP was equivalent to 0.014 ppm in the ration. Application of the method to verification of DBP levels in trout rations from the carcinogenesis study is described. Control ration (0 ppm DBP) was screened for possible DBP contamination and none was found. This is the first report on analysis of DBP in experimental animal diets. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Matrix solid-phase dispersion; Dibenzo[*a*,*l*]pyrene

1. Introduction

Dibenzo[*a*,*l*]pyrene (DBP) is one of the most potent carcinogens known among polycyclic aromatic hydrocarbons (PAHs). It is a potent tumor initiator in rat mammary gland and mouse skin [1–3], and in mouse lung [4]. It is a multi-organ carcinogen in rainbow trout [5]; however, its carcinogenic risk to humans has not been fully assessed. DBP has been detected in cigarette smoke condensate [6], soil and sediment samples [7] and in particulates found by combustion of low sulfur content coal (smoky coal) [8]. When establishing national standards for clean air and water, the Environmental Protection Agency (EPA) must quantify the amount of potential human risk that may be associated with exposure to environmental contaminants such as DBP. However, estimating human risk at one in a million (10^{-6}) is limited by lack of experimental data below a one in 100 (10^{-2}) response. Given the likelihood of human exposure to DBP and the intense regulatory and academic interest in low dose extrapolation, we have conducted a study with DBP and 42 000 rainbow trout to extend the dose response curve for this carcinogen down to

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PII: S0021-9673(01)01207-9

a targeted above-background tumor incidence of 0.1% (10^{-3}) [9]. The study was divided into four quarters and run over a period of 2 years. Each quarter consisted of 10 500 trout, which were fed DBP dietary doses at 0 ppm or ranging from 0.45 to 225 ppm. At the end, the tumor data from all the four quarters were pooled. Considering the complexity of the study, and the importance of feeding the trout the correct dose of DBP, it was vital that a verifiable quality control of the trout diets be maintained. The determination of actual concentrations of the DBP in the diets was essential to this study as these would be used later in modeling the tumor data for risk assessment.

The traditional isolation of fat-soluble analytes [10,11] and, in particular, of PAHs in biological or food matrices [12–14] has consisted of saponification and/or various liquid extractions or partitions followed by one or more clean-up operations on the extract. In an attempt to avoid difficulties associated with these techniques and to speed up sample preparation by reducing the number of lengthy, complicated steps, various alternate approaches have been sought. One of these alternatives is matrix solid-phase dispersion (MSPD) [15] which over the last 10 years has been applied with increasing frequency for the preparation of analytical samples from matrices of biological origin. For example, MSPD has been applied to the analysis of veterinary and agricultural drugs in milk [16] and in animal tissues [17,18], and to pesticides in fruits and vegetables [19,20]. The application of MSPD to lipophilic analytes is of particular relevance to our work, and examples include the analysis of tocopherols and retinyl palmitate in milk-based infant formula [10], alkylphenol ethoxylate non-ionic surfactant biodegradation products from rainbow trout muscle and zebra mussel soft tissues [21], benzo[*a*]pyrene from fish muscle [22] and chlorinated pesticides in beef fat [23].

Our objectives were to develop a method for verifying the levels of DBP present in the semi-synthetic experimental trout diets and to analyze the diets used in the large-scale tumor response study for DBP. The method utilizes MSPD, which is far simpler and more rapid than traditional extraction techniques, to extract and isolate DBP. This is the first report on analysis of DBP in experimental animal diets.

2. Experimental

2.1. Chemicals and materials

Dibenzo[*a*,*l*]pyrene (DBP≥98% by HPLC) and [G-^{3}H] DBP in benzene ([^{3}H]DBP, ≥97%) were obtained from the National Cancer Institute (NCI) Chemical Carcinogen Reference Standard Repository (at Midwest Research Institute, Kansas City, Missouri, USA or at Chemsyn Science Laboratories, Lenexa, Kansas, USA). Benzo[*a*]pyrene (BAP, ~99% by HPLC and GC) was obtained from the NCI Chemical Carcinogen Reference Standard Repository at Midwest Research Institute; BAP (~98% by HPLC) from Fluka, (Milwaukee, WI, USA.) was used in preliminary work. The unlabeled compounds were found to be substantially free from contaminants that could interfere with quantification by the HPLC system described below, and were used without further purification. The [^{3}H]DBP was purified before use by introducing it to a silica Sep-Pak® cartridge (Waters, Milford, MA, USA) and eluting it with *n*-hexane; the resulting material gave a single radioactive spot which co-eluted with the authentic unlabeled material by TLC on Silica Gel 60 precoated plates (EM Separations Technology, Darmstadt, Germany) followed by charring with sulfuric acid or by radioscanning.

DBP and BAP are potent carcinogens that were handled, stored and disposed according to NIH guidelines and Oregon State University procedures – for class C carcinogens. They were protected from light and work was performed under Octron® Gold Filter lamps (OSRAM Sylvania, Danvers, MA, USA). Used glassware was rinsed with CH_2Cl_2, decontaminated with $KMnO_4$–H_2SO_4 according to the published procedure [24,25], and rinsed again before re-use.

Acetonitrile was HPLC grade from EM Science (Gibbstown, NJ, USA), *n*-hexane and dichloromethane were HPLC grade from Mallinckrodt (Paris, KY, USA), and benzene was spectral grade from Fisher (Fairlawn, NJ, USA). Dimethyl sulfoxide

(DMSO, 99.9% HPLC grade) and tetrahydrofuran (THF, 99.9+% HPLC grade, inhibitor free) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All organic solvents were used without further purification. Water was distilled and treated with a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).

2.2. Detection of lipids

In method development, detection of dietary oil lipids was by TLC on silica gel precoated plates with development by *n*-hexane–diethyl ether–glacial acetic acid 80:20:1 and detection by charring with H_2SO_4.

2.3. Standard solutions

Stock solutions of DBP (500 μg/ml) and BAP (1000 μg/ml) for standards were prepared in DMSO, covered with a layer of argon, and stored at below 0°C until use. Working stocks and standard solutions were prepared from the DMSO stocks by dilution with acetonitrile.

2.4. Experimental trout diets

The composition and preparation of the semi-purified Oregon Test Diet (OTD) used in experimental work at our hatchery and rearing facility was as described [26], except that menhaden oil (National Marine Fisheries Service, Charleston, SC, USA) was substituted for salmon oil. DBP was incorporated into the diets by dissolving the appropriate amount of crystalline compound directly in the oil component followed by mixing with the remaining ingredients to form a moist, gelatinous solid. In the carcinogenesis study, diets ‘‘A’’ and ‘‘C’’ through ‘‘I’’ (no diet was designated ‘‘B’’) were designed to contain 0, 0.45, 1.27, 3.57, 10.1, 28.4, 80.0, and 225 ppm DBP, respectively, where ppm is weight (μg) of DBP per dry weight (g) of diet, and where 1 g dry diet is equivalent to 2.857 g wet diet. For development of the analysis method and its validation, we worked at 0.45 (‘‘low’’), 10.1 (‘‘medium’’) and 225 (‘‘high’’) ppm levels.

For some of the method development and recovery studies specified in Results, [^{3}H]DBP was incorporated into diet preparations (~1 μCi ^{3}H/g dry diet). THF or benzene solutions containing [^{3}H]DBP of the appropriate specific activities were prepared. Specific activities were verified by UV and scintillation counting. Solvent from measured volumes of solutions was evaporated with a gentle stream of N_2 before adding the oil. Tritium content of the diets was verified by dissolving 0.1 g aliquots of diet in Solvable™ tissue solubilizer (Packard Instrument Company, Inc., Meridan, CT, USA), adding 0.05 ml glacial acetic acid and 15 ml liquid scintillation cocktail (3a70B™, Research Products International, Mount Prospect, IL, USA) and counting (LS 6500 counter, Beckman Instruments, Fullerton, CA, USA).

2.5. MSPD and sample preparation

Materials and parts used for MSPD and sample extraction were purchased from Varian Sample Preparation Products (Harbor City, CA, USA). These were: Bondesil® EnvirElut™ which is the octadecylsilane (C_{18}) derivatized silica used for MSPD; Mega Bond Elut® 20 cm^3 extraction cartridges with 2 g Florisil®; and 25 mm frit with 20 μm pore size.

Diet aliquots (0.5 g) were placed in a 1-ounce glass mortar containing 2.0 g EnvirElut™. Internal standard (0.5 μg in 0.2 ml for 0.45–10.1 ppm diets, or 10 μg in 1.0 ml for 28.4 through 225 ppm diets) was added to the solid and allowed to sit for 5 to 10 min. For analysis of control diets (0.0 ppm DBP), 0.125 μg BAP internal standard in 0.05 ml was added. The samples were gently mixed for up to ~30 s to produce a semi-moist, homogeneous-appearing mixture. Sample mixtures were then dried overnight in a vacuum desiccator, and poured into the Florisil® extraction cartridges. A frit was inserted and pressed with a syringe barrel onto the top of each sample mixture. The cartridges were placed six at a time, in random order, in a rack and extracted by gravity elution with 30 ml *n*-hexane–benzene, 4:1. Solvent was reduced to near dryness with a rotary evaporator at ≤25°C and samples made to volume (0.5 ml for 10.1 ppm and under or 10 ml for 28.4 ppm and above) with acetonitrile and filtered for HPLC. Standards were prepared at the time of diet mixing

using the same internal standard solutions used for the diets, and standard curves were prepared to cover each of the two dietary DBP concentration ranges specified above. Appropriate modifications to this procedure, specified in Results, were made in method development and recovery experiments.

2.6. HPLC instrumentation and conditions

The HPLC instrumentation consisted of a Baseline 810 (version 3.3) system with two M501 pumps and a SIM module (Waters Corp., Milford, MA, USA), a Spectroflow 980 fluorescence detector (ABI Analytical Kratos Division, The Perkin–Elmer Corp., Norwalk, CT, USA), model 718 autosampler (Alcott Chromatography, Inc., Norcross, GA, USA) and a model CH-30 column heater with model TC-50 controller (Eppendorf Scientific, Madison, WI, USA). The detector parameters were: excitation wavelength, 299 nm; emission filter, 389 nm long-pass; high voltage 744 and rise time 1.00.

The HPLC column was a Symmetry® C_8, 3.9 mm I.D.×150 mm, 5 μm particle size analytical column with a Sentry® C_8, 3.9 mm I.D.×20 mm, 5 μm guard column. The column temperature was 35°C. The mobile phase was 80% acetonitrile–20% water run isocratically at 1.0 ml/min. Sample injection size was 10 μl.

2.7. Statistical comparisons

The Wilcoxon non-parametric rank test was generally used to compare between diets or between diet and standard due to the presence of an outlier, or due to bimodality. In two cases, a two-sided *t*-test was used after removal of an outlier.

3. Results and discussion

3.1. MSPD and extraction of diets

The MSPD technique consists of intimately blending a moist sample with a solid sorbent, most commonly an octadecylsilane derivatized silica, to form a semi-dry, homogeneous-appearing powder. In the mixing process, cellular structures (if present) are disrupted, and lipophilic compounds are solubilized by the C_{18} moiety of the bonded phase. Presumably, the hydrophilic components are oriented on the outer surface of the particles [15,27]. By loading the mixture into an extraction tube containing a second solid sorbent such as unbonded Florisil® or silica, extraction and solid-phase clean-up steps can be performed in a single operation.

The basic semi-synthetic trout diets used in our research consisted of 10% by weight (on dry basis) of menhaden oil. In the preparation of the experimental diets, DBP was dissolved in the oil, which was then added to the casein and other dry ingredients and mixed with water. Recovery of the oil and DBP from diet samples was accomplished by MSPD, which consisted of homogenizing 0.5 g diet sample with 2.0 g EnvirElut™ C_{18} bonded silica. According to the manufacturer of EnvirElut™, its capacity is 5% by weight, or 100 mg, which is much greater than the 18 mg lipid plus analyte encountered in our analytical samples. These sample and sorbent sizes have proved satisfactory for many matrix types including those with a large proportion of lipid [23,28,29].

DBP and lipid were extracted simultaneously from the C_{18} sorbent by mixtures of hexane and benzene, but lipid was readily removed from the extract by retention of the lipid on Florisil® cartridges. By loading the sample–C_{18} sorbent mixtures into a Mega Bond Elut® Florisil® cartridge, the extraction and lipid-removal steps were combined into a single operation. The effectiveness of several hexane–benzene combinations in isolating DBP from the loaded extraction cartridge was examined. Hexane–benzene ratios of 8:2 and 7:3 accomplished the isolation of DBP free from lipid, but at 6:4, small amounts of lipid were co-extracted. The presence of triglycerides and other lipids was undesirable due to their relative insolubility in acetonitrile, the major component of our HPLC mobile phase, and to the potential for extraction of other interferences. We chose to develop the analytical method with the 8:2 ratio in order to assure the absence of lipid from the extract, though the volume of solvent required was somewhat larger than if a lower ratio were used. To optimize penetration of the solvent into the lipid–C_{18} layer, water was removed from the sample–sorbent mixtures under vacuum prior to extraction.

To establish the volume of extraction solvent

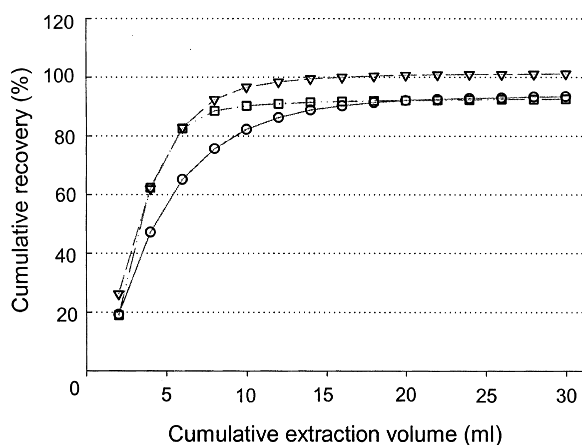

Fig. 1. Elution profiles (in triplicate) for the MSPD extraction of diet containing 0.45 ppm, ~1 μCi/g dry diet [^{3}H]DBP by hexane–benzene 4:1. Profiles for the diets containing 10.1 and 225 ppm [^{3}H]DBP were similar. Different symbols are used for each replicate curve.

required, diets containing [^{3}H]DBP at three concentration levels of DBP (see Experimental) were prepared. During extraction of the diets, 2 ml fractions were collected so that tritium could be determined by liquid scintillation counting. Typical elution profiles of tritium are shown in Fig. 1, where some variability in recovery among samples is evident. In each of three profiles measured at each DBP concentration level, the total solvent volume required to extract 99% of the maximum tritium recovered was 24 ml or less. To assure robustness of the method, we used 30 ml in further method development and validation.

3.2. HPLC

Although separation of DBP and BAP was readily achieved on C_{18} reversed-phase columns, the use of a C_8 solid-phase permitted shorter retention times for DBP (7.8 min at 1 ml/min flow-rate for acetonitrile–water, 8:2) than would have been possible with the corresponding C_{18}. The fluorescence detector was operated at a fixed excitation wavelength (λ_{ex} = 299 nm) rather than at variable wavelengths corresponding to respective absorption maxima for the two compounds to avoid wavelength reproducibility error.

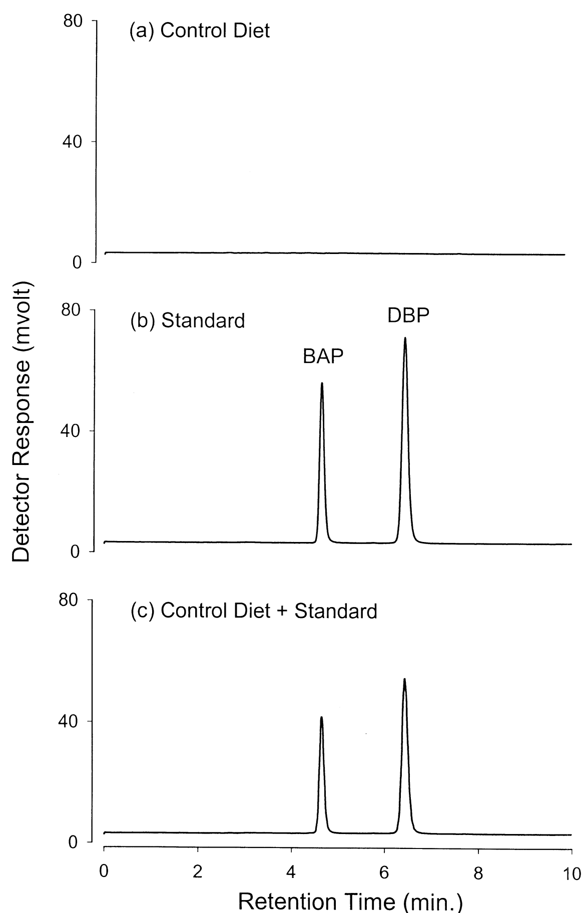

Fig. 2. HPLC chromatograms of (a) extract from control diet; (b) the standard spiking solution containing 2.2 μg/ml DBP (analyte) and 1.0 μg/ml BAP (internal standard); (c) extract from control diet spiked with 500 μl standard in (b) and made to 0.5 ml for HPLC. Fluorescence detector λ_{ex} = 299 nm; emission filter, 389 nm long-pass.

Fig. 2 shows representative chromatograms of extract from control diet, BAP and DBP standards, and of control diet spiked with BAP and DBP. Standard curves were constructed as natural logarithm of DBP/BAP peak area ratio vs. natural logarithm of DBP/BAP concentration ratio to cover the range of peak area ratios and DBP concentrations encountered in the extracts from experimental test diets. Typical estimated parameters and their standard errors obtained from simple linear regressions of the standard data are: intercept = −0.3133 (0.0030) and slope = 1.0051 (0.0025); standard error

for regression = 0.011; $R^2 = 0.9999$, where the data points for the regressions were from triplicate injections at five concentrations of DBP (BAP constant at 1 μg/ml). The coefficients of determination (R^2) for all standard curves were ≥0.9998. Regressions were performed on the log–log scale in order to accommodate the four orders of magnitude for dietary DBP concentration with only two standard curves. By utilizing two dilution levels for the diet extracts (and keeping BAP concentration the same – see Experimental), we were able to keep all DBP concentrations within the detector's linear range.

3.3. Recovery of DBP

Recovery of DBP by the method was determined by analyzing control diet samples that had been spiked with a standard solution containing one of several levels of DBP plus BAP internal standard. The apparent recoveries of analyte in these experiments were calculated by comparing the HPLC DBP/BAP peak area ratios for the extracts with the same spiking solution without extraction. The recoveries, as shown in Table 1, ranged from 100±1 to 102±4% in the first experiment, and from 102+2 to 109±7% in the second. Two batches of control diet were used in both experiments: a freshly prepared control diet ("fresh") and a batch that had been stored at −23°C for up to 2 years ("stored"). The latter control diet was included because of the age of some of the actual diet samples to be analyzed by the method. There was no practical difference in recovery between the two. Further evidence for consistency of DBP recovery was obtained during the application of the method to the analysis of the carcinogenesis study diets. In each of eight diet analysis runs (discussed below), duplicate control diet samples (containing no DBP) were spiked with 0.5 ml of an HPLC standard containing 2.2 μg/ml DBP and 1 μg/ml BAP. The analytical recovery of the spiked DBP (not shown in Table 1) averaged 103±2% (mean±RSD; $n=8$, where each data point is an average for triplicate sample injections of duplicate samples).

In a different experiment, addition of internal standard to the diet extracts rather than to the unextracted diets resulted in analytical recoveries±RSD from 96.7±1.4 to 98.9±1.1% (data not shown). This result is consistent with the above diet spiking experiments and indicates that post-extraction work-up and quantitation losses of DBP relative to the BAP internal standard were minimal.

In the analytical samples, the true extraction efficiency of DBP from the diet matrix with which it is intimately associated may not necessarily be reflected in the usual recovery experiments described above. The semi-synthetic nature of the rations provided an opportunity to assess analyte recovery by incorporating [^{3}H]DBP into a special diet batch prepared for this purpose. Table 2 indicates that the mean extraction recoveries of tritium from the diets in two separate experiments range from 85.3 to 91.0%. Extraction recoveries are relative to the tritium content of the rations without extraction, which was measured separately by direct scintillation counting of diet sample digests. An upper limit on the non-homogeneity of tritium in the diet is estimated by the variability between digest counting samples ($n=10$) which ranged from an RSD of 0.6

Table 1
Analytical recovery of DBP from spiked control diet[a]

DBP conc.[b]	Experiment 1		Experiment 2	
	Fresh diet	Stored diet	Fresh diet	Stored diet
Low	100±0.5	102±3.7[d,e]	109±7.4[f]	107±6.5[f]
Medium	102±0.7[c]	100±1.7	108±5.7[f,g]	107±4.8[f,g]
High	101±0.8	100±1.3	104±0.7[h]	102±2.1[h]

[a] Percent recovery±RSD, where recovery is the DBP/BAP peak area ratio for the extract divided by the mean ($n=6$) peak area ratio for the standard analyte/i.s. solution used to spike the control diet sample before extraction. RSD accounts for variation in test diet sample but not for variation in the standard solutions. Diet sample size $n=5$.

[b] "Low" = 0.45 ppm, "Medium" = 10.1 ppm, "High" = 225 ppm DBP in diet.

[c] Recovery is significantly different from 100% ($P=0.004$, non-parametric Wilcoxon test).

[d] Statistical difference between "fresh" and "stored" diet is ambiguous. With all data, $P=0.15$ (Wilcoxon test), but with one outlier (=108%) excluded, $P=0.002$ (two-sided t-test).

[e] Recovery difference from 100% is ambiguous. With all data, $P=0.18$ (Wilcoxon test), but with one outlier (=108%) excluded, $P=0.002$ (two-sided t-test).

[f] Bimodal distribution for the five samples (two samples at approximately 100% and three samples ≥111%).

[g] Some evidence ($P \leq 0.05$, Wilcoxon test) that median recovery is greater than 100%. Note bimodal distribution described above.

[h] Recovery is significantly different from 100% ($P=0.004$, Wilcoxon test).

Table 2
Extraction recovery of tritium from diets containing [^{3}H]DBP

Concentration of DBP	Experiment	Diet replicate	Percent recovery[a] mean±SD
"Low" (0.57 ppm)[b]	A	1	89.6±0.9[c]
"Low" (0.57 ppm)[b]	A	2	89.5±1.3[d]
"Medium" (10.1 ppm)	A	1	86.1±1.0[c]
"Medium" (10.1 ppm)	A	2	85.3±0.6[c]
"Medium" (10.1 ppm)	B	1	91.0±0.6[e]
"Medium" (10.1 ppm)	B	2	89.9±0.9[e]
"Medium" (10.1 ppm)	B	3	90.1±2.0[e]
"High" (225 ppm)	A	1	89.3±0.5[c]
"High" (225 ppm)	A	2	88.2±1.5[c]

[a] Extraction recoveries are relative to the tritium content of the diets without extraction, which was measured by digestion and scintillation counting.
[b] "Low" concentration was not 0.45 ppm due to limits imposed by specific activity of [^{3}H]DBP.
[c] Sample size: $n=5$.
[d] Sample size: $n=4$.
[e] Sample size: $n=6$.

to 1.7% for nine different diet preparations. The variability indicates the effectiveness of the diet mixing process. These recovery measurements are considered to be more reliable than those that could be calculated from the elution profile data (Fig. 1) due to the possibility of cumulative errors in the latter. Aliquots of the collected eluates were counted directly, so that any losses of DBP reflect limitations of the extraction process and do not include possible losses in post-extraction work-up. The analytical recoveries and precision compare favorably with other analyses for PAHs in food or biological tissue [13,14,30,31] including one that utilizes MSPD [22]. The tritium recovery experiments (Table 2) indicate an overall extraction efficiency of 85.3–91.0%, yet spiking experiments (Table 1) gave recovery ≥100%, indicating that the internal standard is effective in compensating for extra-matrix extraction, work-up, and HPLC losses. There is some small inconsistency between the first and second spiking experiment (Table 1), as recoveries are as great as 109% in the second experiment, and variabilities are twice the magnitude of those in the first. The data of the second experiment form a bimodal distribution that could be explained by an erratic loss of internal standard that is not matched by loss of analyte. Such losses of BAP and other PAHs have been observed by others in the lengthy traditional methods and have been attributed mainly to photo-degradation, oxidation by O_2 or by peroxides in ethers, volatilization of low molecular mass PAHs, and to irreversible binding to Florisil® or silica clean-up columns [13,14]. In the present study, steps were taken, where applicable, to minimize their effects (see Experimental).

3.4. Analysis of DBP in carcinogenesis study diets

The carcinogenesis study [32] required that eight sets of diets consisting of eight different DBP concentrations per set (0, 0.45, 1.27, 3.57, 10.1, 28.4, 80, and 225 ppm DBP) be prepared over a period of nearly a year. A complete diet set was processed in a single analytical run, with four replicate samples taken at each DBP level (except that the "0" ppm control diets were screened in a separate run). Also included were a standard curve for each of two dietary DBP concentration ranges (see Experimental), duplicate control diet blanks (to verify absence of significant interferences), and duplicate control diet spiked with a standard (2.2 μg/ml DBP, 1.00 μg/ml BAP, verifies recovery). The results are given in Table 3. The mean analytical ppm concentrations are lower than the target values by an increasing percent as target ppm increases, so that diet C (target 0.45 ppm) was 3.3% below target, diet F (target 10.1 ppm) was 5.8% below, and diet I (225 ppm) was 11.4% below. Neither the tritium-recovery experiments (Table 1) nor the spiking experiments (Table 2) completely explain this trend, though factors involved in the bimodal effect observed in experiment 2 (Table 2) could have a role. A more detailed statistical analysis of the diet analysis data will be made when the data are incorporated into the modeling for the carcinogenesis study [32].

Table 3
Analysis of DBP in trout experimental rations

Diet	Target DBP (ppm)	Found DPB (ppm) Mean±%RSD[a]
C	0.45	0.44±2.5
D	1.27	1.22±3.8
E	3.57	3.45±5.8
F	10.10	9.51±4.6
G	28.40	26.10±4.3
H	80.00	72.90±4.2
I	225.00	199.00±3.6

[a] $n=8$.

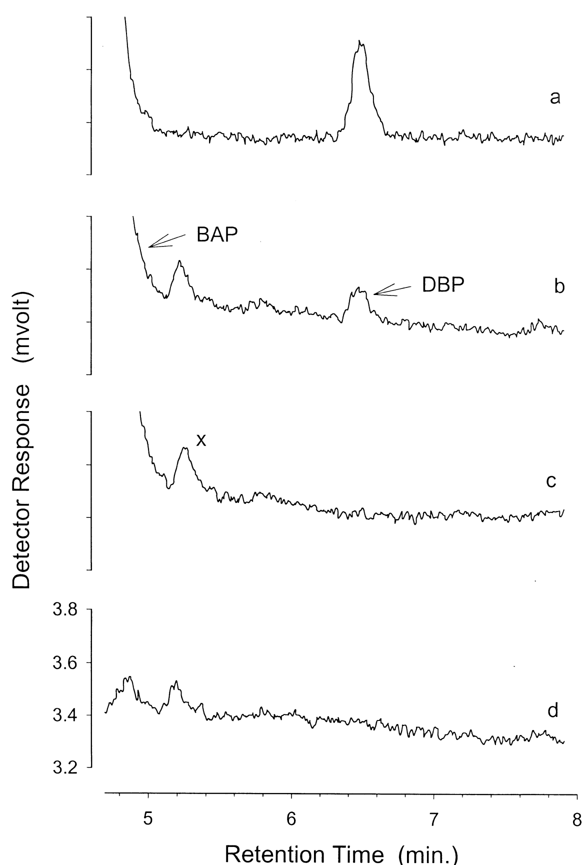

Fig. 3. HPLC chromatograms from screening of control diets ("diet A", made as 0 ppm DBP) for DBP contamination. (a) A standard containing 5.00 ng/ml DBP and 250 ng/ml BAP internal standard. (b) Extract from control diet spiked with 2.50 ng DBP and 125 ng BAP. (c) Extract from control diet spiked with 125 ng BAP only. (d) Extract from 0.5 g control diet without spiked DBP or BAP. Diet samples were 0.5 g, and final extract volumes were 0.5 ml (see Experimental). Units on all detector response axes are the same as for chromatogram (d). The source of the peak marked "x" (seen in b–d) is the control diet. Fluorescence detector settings are the same as for Fig. 2.

Screening of "0" DBP control diet (diet A) to verify the absence of detectable DBP was performed separately from the DBP-containing diets (Fig. 3). For this purpose, a separate standard curve covering the range 0.01 to 0.5 μg/ml DBP, with 0.25 μg/ml BAP was constructed on the natural log scale. The curve appeared to be linear and had estimated parameters (± SE): intercept = −0.2861 (0.0078); slope = 1.0141 (0.0039); SE for the regression was 0.025; R^2 = 0.9996. Control diets spiked with DBP at several levels including the estimated lowest limit of detection (LLD) were included in the analysis to verify the recovery of DBP. The LLD, defined as three times the peak to peak baseline noise, was estimated to be approximately 0.005 μg/ml. This corresponds to a dietary concentration of 0.014 ppm, which was over 30 times lower than the lowest dietary DBP concentration (0.45 ppm) used in the carcinogenesis study. The analytical method was not optimized for maximum sensitivity because the LLD (0.014 ppm) was found to be adequate for the purpose of the study. All control diets from the study were analyzed, but no DBP was detected in any, as exemplified in Fig. 3.

4. Conclusions

An MSPD-based method for the extraction and quantification of DBP from experimental diet samples has been developed. The simplicity of the method is a consequence of combining the extraction and clean-up procedures in a single step, and of the relatively small amounts of solvent and sample required. Contact time of the sample with Florisil® is of the order of minutes, and no UV light is required to monitor the elution of the sample, hence several possible sources of degradation or sample loss are minimized or avoided. The method can be readily applied in a laboratory where minimal personnel and equipment are available. Method performance is comparable to approaches that are more traditional.

The method, when applied to experimental trout diets covering a 500-fold range of DBP, provided measured DBP concentrations that were 97% (lowest dose) to 89% (highest dose) of targeted DBP concentrations. Results from the spiking and tritium-recovery experiments indicate that the discrepancies in trout diet recoveries are not due to dose-related bias in the analytical method.

Acknowledgements

We thank the staff at the Food Toxicology and Nutrition Laboratory for expertise and help with the diet preparations. This is technical paper no. 11763 from Oregon Agricultural Experiment Station. This work was supported by grants ES 04766, ES 03850, and ES 00210 from the National Institute of En-

vironmental Health Sciences, NIH, US Public Health Service.

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